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INFLUENCE OF PROTEIN CONTENT ON THE ABSORPTION OF ANTITOXIN AND AGGLUTININ INJECTED SUBCUTANEOUSLY.*

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The subject of this communication has become important because of the fact that diphtheria and tetanus antitoxins are supplied in this country most commonly in the form of the refined and concentrated globulin preparation. The methods employed in the United States are those developed in this laboratory by Atkinson, Gibson and Banzhaf. These antitoxic preparations contain that fraction of the serum protein designated pseudoglobulin. In the actively immunized horse, the antitoxins are intimately associated with the pseudoglobulin, and by the process of "salting out" may be separated with it from the other proteins, such as albumin, euglobulin, etc. By these methods, a concentrated preparation of diphtheria antitoxin, for instance, may be prepared containing 2,000–3,000 units per cubic centimeter with a protein (pseudoglobulin) concentration of 18–20 per cent, starting with an original serum containing 400–600 units and a protein content of 8–9 per cent. This concentration of antitoxin is obtained about equally by the elimination of the non-antitoxic proteins and by the abstraction of water. The removal of the water is merely a convenience, while the removal of the proteins, together with the changes produced by the heating employed in the process, removes certain deleterious substances which are present in the whole serum. Careful observation of cases in the hospital wards under our supervision has shown that serum reactions are less frequent in patients receiving the refined antitoxin than in those who have had the whole serum product.

In the treatment of diphtheria, it is a well recognized fact that the antitoxin should reach the general circulation as quickly as possible after its administration. The injection of the antitoxin

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directly into the blood stream is the only way that fully meets such requirements, but owing to the technical difficulties encountered, this method is not commonly employed by the practitioner. Instead, the subcutaneous injection is the means of administration in general use. In connection with the subcutaneous method of administration, this question has arisen: Does the highly concentrated antitoxic preparation, with its increased protein content, pass as rapidly from the subcutaneous or muscular tissues to the blood as do preparations having a larger volume of fluid of lower protein and lower antitoxin concentration? This question becomes of special interest in connection with certain commercial preparations of concentrated antitoxin containing a percentage of protein sometimes reaching $3\frac{1}{2}$ times that of the native antitoxic serum.

The influence on absorption of the protein concentration in the antitoxin solutions was considered by us when we began to use the refined antitoxin. The results of our investigations convinced us that a concentration to at least double that of an immunized horse serum had little, if any, appreciable effect in retarding absorption. The results obtained by L. E. Walbum[1] with the absorption of agglutinin in rabbits which showed that, in those animals in which the protein concentration of the injected fluid was equal to that of the blood serum, the agglutinin was more rapidly absorbed than from solutions containing either a higher or lower protein concentration caused us to attempt a new investigation of the subject.

Walbum states at the beginning of his paper that on account of work carried out by Henderson-Smith and E. Tobin he assumed that the conditions affecting the absorption of antitoxins were similar to those affecting the absorption of agglutinins. He went even farther and assumed that the conditions affecting the absorption in the subcutaneous tissues of rabbits of *B. coli* agglutinin produced by the goat and contained in goat serum or goat globulins were similar in the case of the absorption of horse-produced antitoxin from the tissues of man. In somewhat condensed form, his conclusions are as follows: The absorption of agglutinin from the subcutaneous tissues is much slower from serum containing 15 per cent of protein than from that containing the normal amount (6.2 per cent, goat). When the more concentrated protein solution is diluted with normal saline solution, so as to be reduced to the same protein content as the normal, the agglutinin is absorbed equally from both solutions. Less than the normal concentration retards the absorption from subcutaneous tissues, but increases the rate of absorption from that injected into the muscles. The absorption from the peritoneal cavity is not affected by the protein concentration of the serum.

A critical examination of Walbum's results gives the impression that the difference in rapidity of absorption of the agglutinin from the solutions of different protein concentrations was less marked than Walbum judged. Thus, if the rabbit receiving the concentrated protein in his Experiment 1 is compared with the rabbit receiving the normal protein percentage in the Experiment 7, the absorption curves are practically identical, if allowance is made for the different weights of the animals. The similarity of the rate and degree of absorption of the agglutinin from solutions of different protein concentrations is strikingly shown in Walbum's Experiment 10. We reproduce his Table 1, simply adding figures in brackets correcting the amounts to correspond with a weight of 1,950 grams.

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[1] *Ztschr. f. Immunitätsf.*, 1912, 12, p. 546.

The figures in this table show that in this experiment the increased protein concentration had no appreciable effect in retarding the rate of absorption or the amount. While the lessened protein concentration appeared to decrease the rate and amount of absorption after the first day, yet it apparently increased both in the earlier and more important period.

TABLE 1.

VERSUCH X. SUBKUTANE INJEKTION (WALBUM).

	14,95 PROZ. EIWEISS KANINCHEN, 1,950 G.		10 PROZ. EIWEISS KANINCHEN, 1,950 G.		7 PROZ. EIWEISS KANINCHEN, 1,700 G.		4 PROZ. EIWEISS KANINCHEN, 1,700 G.		1 PROZ. EIWEISS KANINCHEN, 1,750 G.	
		Aggl.-Einh. in 1 c.c. Ser.		Aggl.-Einh. in 1 c.c. Ser.		Aggl.-Einh. in 1 c.c. Ser.		Aggl.-Einh. in 1 c.c Ser.		Aggl.-Einh. in 1 c.c. Ser.
Vor der Injektion....	>0,2		>0,2		>0,2		>0,2		>0,2	
16 Std. nach der Injekt.	0,03	33,3	0,0225	44,4	0,0185	54 [47]	0,015	67 [58.4]	0,013	77 [69.1]
26 Std. nach der Injekt.	0,007	143	0,007	143	0,007	143 [124.6]	0,005	200 [174.3]	0,007	143 [128.3]
40 Std. nach der Injekt.	0,0035	286	0,003	333	0,0029	340 [296.4]	0,004	250 [217.9]	0,0055	182 [163.3]
50 Std. nach der Injekt.	0,0029	345	0,00275	364	0,0025	400 [348.7]	0,0037	270 [235.3]	0,004	250 [224.3]
64 Std. nach der Injekt.	0,0025	400	0,00225	444	0,0021	476 [414.9]	0,0035	286 [249.3]	0,0037	270 [242.3
74 Std. nach der Injekt.	0,0026	384	0,0022	455	0,002	500 [435.8]	0,0035	286 [249.3]	0,0037	270 [242.3
88 Std. nach der Injekt.	0,00275	364	0,00225	444	0,0021	476 [414.9]	0,0037	270 [235.3]	0,004	250 [224.3]
98 Std. nach der Injekt.	0,004	250	0,0025	400	0,0024	417 [363.5]	0,005	200 [174.3]	0,0049	204 [183]
136 Std. nach der Injekt.	....	....	0,004	250	0,0035	286 [249.3]	....	....	....	....

ORIGINAL INVESTIGATIONS.

As it is possible that the sera of different animals vary in their absorption characteristics, we determined to test the effect of protein concentration upon absorption by using the serum of the horse because this animal is almost universally employed to furnish protective sera for man. Because of the importance of this question and the lack of danger of the experiment, we also tested the matter in man as well as in animals.

Our first experiments to test the question of absorption of antitoxin in different protein concentrations were performed upon goats. Nine animals, which were divided into two main groups, were selected for the tests. Serum samples taken for controls before beginning the experiments showed no appreciable amounts of antitoxin. Each animal received 10,000 units of diphtheria antitoxin by subcutaneous injection in the lower abdominal region. The antitoxin given was incorporated with widely different concentrations of protein (pseudoglobulin), and sufficient NaCl was added to make isotonic (0.8 per cent). Different volumes of the mixtures also were used in the injections. Two animals received a volume of 2.7 c.c. containing 30 per cent solids; 3 animals, 10 c.c. volume, 30 per cent solids; while one received 30 c.c. volume, 30 per cent solids. Three animals received solutions having only 7.5 per cent total solids. As mentioned above, each mixture contained 10,000 units of diphtheria antitoxin, and in 6 of the cases the animals were injected in one place only. While in the remaining 3 instead of injecting in one place, the dose was equally distributed in 3 places in each animal. Blood samples taken from each animal at intervals after the injection had the serum removed by clotting out, and the antitoxic value of each was determined for the following periods: 18, 36, 48, 72, and 96 hours. As a basis for comparison the antitoxic value in units per cubic centimeter was computed relative to an animal weighing 50 lbs. and the results tabulated as shown in Table 2.

TABLE 2.

INFLUENCE OF PROTEIN CONCENTRATION UPON ABSORPTION OF ANTITOXIN.
GOATS (SUBCUTANEOUS INJECTION).

	10,000 UNITS DIPHTHERIA ANTITOXIN								
	Solids, 30 Per Cent (Protein, 29.2 Per Cent+NaCl, 0.8 Per Cent)						Solids, 7.5 Per Cent (Proteins, 6.7 Per Cent+NaCl, 0.8 Per Cent)		
Number.......	1 H.	2 H.	3 H.	4 H.	5 H.	6 H.	1 L.	2 L.	3 L.
Volume injected	30 c.c.	10 c.c.	10 c.c.	10 c.c.	2.7 c.c.	2.7 c.c.	10 c.c.	10 c.c.	10 c.c.
Places injected..	3	3	1	1	1	1	3	1	1
After injection	Units	Units	Units	Units	Units	Units	Units	Units	Units
18 hrs.......	7.82	7.86	6.8	5.7	8.37	5.58	8.22	8.75	7.68
36 “	10.2	9.07	8.83	7.6	8.64	6.97	10.72	9.0	8.4
48 “	10.2	8.47	9.12	7.6	8.91	7.16	10.01	8.5	7.92
72 “	8.84	8.47	9.12	7.6	8.64	6.51	9.65	8.0	7.2
96 “	6.8	6.05	7.41	5.7	7.56	6.51	7.86	6.0	7.2

The antitoxic unit strength was first estimated between points of 0.25 units difference. In correcting the figures to a uniform goat weight (50 lbs.) the figures show apparently more accurate estimation.

The antitoxic unit strength was first estimated between points of 0.25 unit difference. In correcting the figures to a uniform goat weight (50 lbs.) the points of estimation necessarily show different intervals. The weights of the animals were as follows, given in order from left to right: 68, 60.5, 57, 47.5, 54, 46.5, 71.5, 50, and 48 lbs., respectively.

Owing to individual irregularities in absorption, the average values were taken for all the animals which received antitoxin with low solid content, and for those which received antitoxin with high solids, and comparisons were made between the two series (Chart 1). It was found that the degree of absorption was somewhat greater for the low solid content mixtures throughout, especially the first period (18 hrs.), in which it was approximately 15 per cent higher; this difference gradually diminished so that at the thirty-sixth hour period, it was less than 5.5 per cent, and at 48 hours, 2.7 per cent.

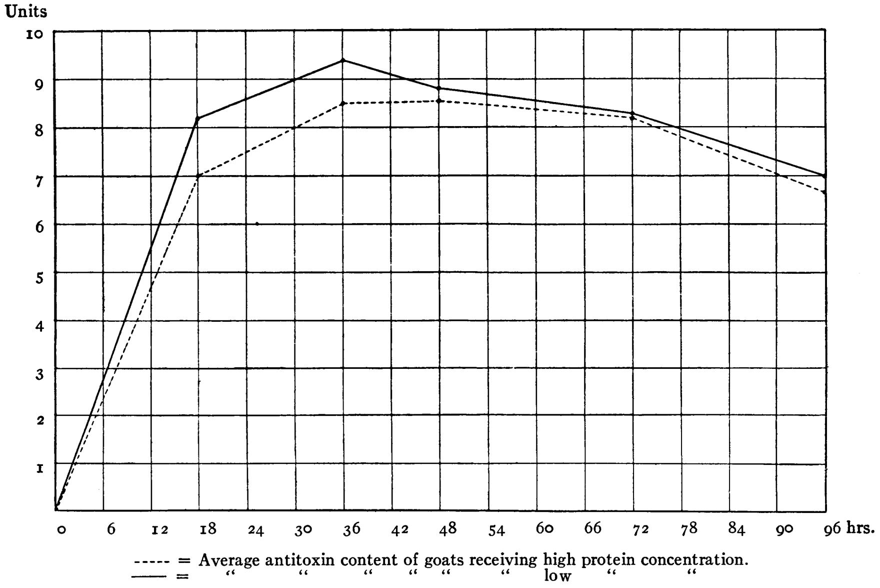

CHART 1.—Comparative absorption from high and low protein concentrations.

A second series of tests were carried out upon normal men. Two men received subcutaneous injections of 4.5 c.c. diphtheria antitoxin containing 10,000 units and 18 per cent of total solids. Two men were given the same dose of antitoxin (10,000 units) to which an equal volume of physiological salt solution had been added, thus doubling the volume (9 c.c.) and reducing its protein percentage one-half. The dose was injected subcutaneously in one place. Bleedings were taken at definite intervals after the injection. The antitoxin content was determined for each blood sample, then the unit value per cubic centimeter was computed to a standard weight (100 lbs.) for comparative purposes. Slight amounts of normal antitoxin were subtracted, when present, for purposes of tabulation. These results are shown in Table 3.

TABLE 3.

Men (Subcutaneous Injections).

Injected in One Place	10,000 Units Diphtheria Antitoxin			
	Solids, 18 Per Cent (Protein, 17.2 Per Cent+NaCl, 0.8 Per Cent)		Solids, 9.4 Per Cent (Protein, 8.6 Per Cent+NaCl, 0.8 Per Cent)	
Subject..........	R.	S.	J.	F.
Volume injected....	4.5 c.c.	4.5 c.c.	9 c.c.	9 c.c.
After injection	Units	Units	Units	Units
3 hrs..........	0.45	0.52−	0.12+	0.27+
6 "	0.72	0.91	0.25	0.55
9 "	1.17	1.04	0.31	0.82
24 "	2.70	3.38	0.93	1.86
48 "	3.15	4.03	2.0	2.76
96 "	3.15	4.29	2.0+	2.76−

The unit value of each serum sample in this table has been computed to a standard weight of 100 lbs. for purpose of direct comparison. Weights of men, reading from left to right, are 180, 260, 125, and 138 lbs., respectively.

In these experiments it was found that when the weight was considered the degree of absorption was greater in the men who received the concentrated preparation. This is the opposite of the findings in the tests on goats. Considerable variation of weight existed between the different men, the extremes being 125 lbs. and 260 lbs. Believing that the weight factor and other individual characteristics influenced the foregoing result, it was decided to reinject two of the men. Two of the subjects, one large and one small, were selected from the group and used for reinjection. About 9 weeks after their first injection each one received a second subcutaneous injection (in one place) of 10,000 units of diphtheria antitoxin from the same lot as used in the first series of tests. But, in this case the smaller man instead of the larger received the 4.5 c.c. volume of 18 per cent total solids preparation. Blood samples were taken from each and the antitoxic content of the serum was determined.

Upon comparison, it was found that the absorption of antitoxin in each man corresponded very closely to the results shown in the first test (Table 4). Therefore, it appears that in this series of tests, the rate and degree of absorption were largely independent of the percentage of protein present within the limits used, but were individual characteristics depending upon the subject receiving the injection. The persistence of the antitoxin in the blood is not affected by the protein concentration.

TABLE 4.

MEN (SUBCUTANEOUS INJECTIONS).

INJECTED IN ONE PLACE	10,000 UNITS OF DIPHTHERIA ANTITOXIN			
Subject...........	F.		S.	
Volume injected....	Second Injection 4.5 c.c.	First Injection 9.0 c.c.	First Injection 4.5 c.c.	Second Injection 9.0 c.c.
Concentration	Solids, 18 Per Cent (Protein, 17.2 Per Cent+NaCl 0.8 Per Cent)	Solids, 9.4 Per Cent (Protein, 8.6 Per Cent+NaCl 0.8 Per Cent)	Solids, 18 Per Cent (Protein, 17.2 Cer Pent+NaCl 0.8 Per Cent)	Solids, 9.4 Per Cent (Protein, 8.6 Per Cent+NaCl 0.8 Per Cent)
After Injection: (Hrs.)	Units	Units	Units	Units
24............	1.86	1.86	3.38	3.12
48............	2.96	2.76	4.03	3.64
72............	2.82	....	....	3.9
96............	2.82	2.76	4.29	3.9

High and low protein concentrations in the same individuals.

After completing the series of experiments upon man and goats, we decided to perform similar experiments upon rabbits, using a specific agglutinin, thus taking a type of antibody and species for test animal which would allow us to compare our results with those of Walbum. A specific agglutinin for *B. typhosus*, derived from an immunized horse, was fractionated and the pseudoglobulin separated with a portion of the agglutinin; this, after dialysis, was further concentrated in a desiccator over H_2SO_4 at 36–37° C. The concentrated product contained 20 per cent solids, including 0.8 per cent NaCl, and had double the agglutinating value of the original agglutinating serum. The original agglutinating serum contained 9.88 per cent total solids. Three groups of 3 animals each were given subcutaneous injections in one place (over the abdomen) as follows: (1) 16 c.c. original agglutinating serum (low solids); (2) 8 c.c. volume, concentrated agglutinin preparation (high solids); (3) 16 c.c. volume, composed of 8 c.c. concentrated agglutinin preparation, 8 c.c. normal pseudoglobulin containing the same percentage of total solids. Bleedings were made at intervals after injection, and the usual quantitative tests for agglutinins were made upon the clotted-out serum.

TABLE 5.

Rabbits (Subcutaneous Injections)

Typhoid Agglutinins 80,000 Units	Group 1			Average	Group 2			Average	Group 3			Average
Weight of Animals	1,560 gm.	1,570 gm.	1,500 gm.	1,543 gm.	1,550 gm.	1,570 gm.	1,510 gm.	1,543 gm.	1,530 gm.	1,600 gm.	1,485 gm.	1,538 gm.
Agglutinin Preparation	Low Solids	Low Solids	Low Solids		High Solids	High Solids	High Solids		High Solids	High Solids	High Solids	
Volume	16 c.c.	16 c.c.	16 c.c.		8 c.c.	8 c.c.	8 c.c.		16 c.c.	16 c.c.	16 c.c.	
Local Reaction	Very Slight	Very Slight	Very Slight		Marked	Moderate	Marked		Marked	Intense	Intense (Abscess)	
After Injection: (Hrs.)	Aggl. U.	Aggl. U.	Aggl. U.		Aggl. U.	Aggl. U.	Aggl. U.		Aggl. U.	Aggl. U.	Aggl. U.	
6	60	70	60	63	40	60	40	46	30	40	10	26
12	120	145	120	128	80	135	100	105	60	90	40	63
24	200	245	250	231	150	235	205	196	150	170	90	136
36	250	345	350	315	200	335	280	271	250	240	125	205
48	300	420	400	373	275	385	330	330	300	315	150	255
60	350	470	450	423	300	415	380	365	350	340	175	288
75	400	520	400	440	350	485	380	405	400	365	200	321
100	325	470	350	381	325	460	330	371	350	390	225	321

The average weight of the rabbits comprising each of the 3 groups being practically identical, the measure of the agglutinating strength of the serum taken from the individual rabbits has not been corrected for a common weight. Instead the average strength of each group has been added.

In those animals which received the concentrated preparation, a local reaction occurred at the point of injection, followed by infiltration, and, in several animals, a necrosis of the surrounding tissues. As is evident, this reaction greatly disturbed the normal absorptive function. However, it may be of interest to note that when the results of the tests were averaged for each of the 3 groups (Table 5) and curves plotted, they roughly paralleled each other, each group simply showing different degrees of absorption. The first group of animals which received the original agglutinating serum gave only a slight local reaction and showed the highest degree of absorption of the specific agglutinin. The second group which received 8 c.c. of the concentrated preparation showed less absorption throughout, while the third group with the 16 c.c. volume high solids was still lower. These results would indicate that an infiltration about the injected area must also be an important factor in retarding the absorption of antibodies. The latest period at which the tests were taken revealed that the different sera had approached each other in agglutinating strength. There was slight bacterial contamination of the injected sera. It is not thought that this influenced the local reaction, but it is possible that it did.

CONCLUSION.

The degree of protein concentration which is usually employed to produce the refined and concentrated diphtheria antitoxic globulin preparations has little or no effect in retarding the absorption of the antitoxin from the subcutaneous tissues. The removal of water, if not pushed too far, is therefore a justifiable means of lessening the quantity of fluid to be injected.

Any preparation which causes local inflammatory reaction lessens the rate of antitoxin absorption.